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Antizyme inhibitor is rapidly induced in growth-stimulated mouse fibroblasts and releases ornithine decarboxylase from antizyme suppression

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Ornithine decarboxylase (ODC) catalyses the first step in the synthesis of the polyamines putrescine, spermidine and spermine. The polyamines are essential for cell growth, but at elevated levels they may be tumorigenic, toxic, or may induce apoptosis. Therefore, ODC activity is highly regulated. It is induced when cells are stimulated to grow, and it is subjected to feedback inhibition by the polyamines. By causing ribosomal frameshifting, polyamines induce the synthesis of antizyme, a 23-kDa protein, which binds to ODC, inhibits its activity and promotes its degradation by the 26 S proteasome. Antizyme, in turn, is inhibited by antizyme inhibitor (AZI). We describe the cloning of a mouse AZI cDNA, encoding a protein with high homology to mouse ODC. Using purified recombinant proteins, we show that AZI (which has no ODC activity) can release enzymically active ODC from antizyme suppression *in vitro*. We also show that ODC reactivation takes place in mouse fibroblasts upon transient transfection with an AZI-expressing plasmid construct. Finally we demonstrate that the AZI mRNA content of mouse fibroblasts increases significantly within an hour of growth stimulation, i.e. much earlier than ODC transcripts. Our results indicate that induction of AZI synthesis may represent a means of rescuing ODC molecules that have been inactivated and tagged for degradation by antizyme, when culture conditions improve and polyamine production is needed for cell growth and proliferation.

Key words: polyamines, ribosomal frameshifting, serum stimulation.

INTRODUCTION

Ornithine decarboxylase (ODC) is a key enzyme in the biosynthesis of the polyamines putrescine, spermidine and spermine. The polyamines are essential for growth and differentiation of mammalian cells, and their synthesis, catabolism and transport are highly regulated [1]. ODC gene expression is stimulated by various mitogens [2–5]. Since elevated ODC activity is seen in tumour cells, and overexpression of ODC can lead to neoplastic transformation, the means by which the enzyme is regulated have attracted much attention [6–10]. The polyamines themselves feedback-regulate ODC, mainly at the translational and post-translational levels [11].

Post-translational regulation of mammalian ODC has been attributed to the action of antizyme, a small protein whose synthesis is stimulated by high polyamine levels [12,13]. Antizyme inactivates and targets ODC for rapid degradation by the 26 S proteasome. The antizyme protein is encoded by two open reading frames (ORFs) in the mRNA, where the second ORF (ORF2) is in +1 frame relative to the first ORF (ORF1). Polyamines and agmatine (a decarboxylation product of arginine) induce a ribosomal frameshift from ORF1 to ORF2, thus making possible the generation of a full-length antizyme protein [14–16]. Antizyme mRNA was the first mammalian gene product found to be dependent on a translational frameshift mechanism for proper decoding. We have shown previously that the transcription of the antizyme gene decreases when cells are depleted of polyamines, indicating that the polyamines are involved in maintaining the basal transcription rate of the gene [17]. Besides tagging ODC for degradation, antizyme inhibits the uptake of polyamines by affecting the polyamine transporter [18–20]. Recently, a new member of the antizyme family has been discovered [21]. It also requires +1 ribosomal frameshifting for its synthesis, binds to ODC and inhibits polyamine transport [22]. However, the gene of this new family member is expressed to a much lower extent [21].

Yet another factor, antizyme inhibitor (AZI), is involved in the regulation of ODC. This protein has a higher affinity for antizyme than ODC [23–25]. AZI can therefore rescue ODC from its complex with antizyme, and prevent ODC from being degraded rapidly by the 26 S proteasome. The recent cloning and sequencing of rat and human cDNAs encoding AZI ruled out the possibility that AZI was merely a product of post-translational ODC modification [26,27]. Thus AZI has a unique sequence, although closely related to that of ODC. ODC has to dimerize in order to form an active site [28]. AZI also forms a homodimer, but this dimer lacks ODC activity. Despite their resemblance, AZI and ODC monomers do not form heterodimers [26]. AZI protein expression is induced in the liver when rats are treated with thioacetamide, and the AZI mRNA content increases in the heart upon isoprenaline treatment [23,27].

The aim of the present study was to elucidate the regulation of ODC by antizyme and AZI in mouse cells. To obtain the missing tool, we cloned and sequenced a mouse AZI cDNA. By expressing AZI cDNA as well as cDNAs encoding ODC and antizyme in bacteria, large amounts of the corresponding proteins were produced and then purified. In experiments where these recombinant proteins were mixed *in vitro*, or their corresponding cDNAs were expressed in mouse fibroblasts (following transient transfection), the regulation of ODC activity was analysed. Our

Abbreviations used: AZI, antizyme inhibitor; EST, expressed sequence tag; FCS, fetal calf serum; GS-E, *N*-(3-methoxy-2-ethylbenzoyl)-*N'*-(3,5-dimethylbenzoyl)-*N'*-t-butyl hydrazine; ODC, ornithine decarboxylase; ORF, open reading frame; UTR, untranslated region.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AF032128.

results indicate that AZI is an important secondary regulator, capable of preventing and rescuing ODC from degradation initiated by binding to the primary regulator, antizyme.

MATERIALS AND METHODS

Materials

All radiochemicals were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Oligonucleotides for cloning and sequencing were from DNA Technology (Aarhus, Denmark). The ecdysone agonist *N*-(3-methoxy-2-ethylbenzoyl)-*N'*-(3,5-dimethylbenzoyl)-*N'*-t-butyl hydrazine (GS-E) and the antibiotic Zeocin were from Invitrogen (Groningen, The Netherlands).

Cells

Swiss 3T3 mouse fibroblasts were grown routinely in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum (FCS). EcR-3T3 mouse fibroblasts (Invitrogen), generated by stably transfecting a plasmid (pVgRXR), carrying a modified ecdysone receptor, into NIH 3T3 cells, were grown in the same medium supplemented with 10% FCS and 400 μg/ml Zeocin. Balb/c 3T3 mouse fibroblasts, on the other hand, were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS. All cells were seeded at a density of 2×10^4 cells/ml.

For serum starvation, Swiss 3T3 cells were grown to confluency and then maintained for 24 h in medium containing 0.2% FCS. For serum starvation, EcR-3T3 cells were grown to subconfluency and then maintained for 24 h in medium containing 0.1% FCS. Swiss 3T3 and EcR-3T3 cells were stimulated to grow by the addition of medium containing 10% FCS. To serum-starve Balb/c 3T3 cells, they were first grown to confluency and then maintained for 48 h in medium containing 0.2% FCS. To stimulate their growth, medium containing 20% FCS or 40 nM PMA, was added.

Cloning and sequencing of a mouse AZI cDNA

RNA was extracted from Balb/c 3T3 cells as described below, and first-strand synthesis was performed using oligo-dT_{15} primers and dNTPs from Pharmacia and reverse-transcription buffer and Superscript from Gibco-BRL. The DNA in an aliquot of the first-strand-synthesis reaction mixture was amplified by PCR using 2.5 units of KlenTaq polymerase (Clontech), 0.2 mM dNTP and 20 pmol of each primer in PCR buffer (Clontech). The sense primer (5'-GTGGAATACGGCTGAGATG-3') was derived from the rat AZI cDNA sequence (accession number D89983), and the antisense primer (5'-TTAAGGGGTGG-GTCAAAC-3') was derived from a mouse expressed sequence tag (EST) clone (accession number AA162795). The PCR was carried out in a thermal cycler under the following conditions: 2 min of denaturation at 94 °C and 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 90 s. The PCR product was purified using QiaQuick PCR purification kit and ligated into pGEM-T (Promega), using Ready-to-go ligase (Pharmacia), according to recommendations from the manufacturer. One-tenth of the ligase reaction was used to transform competent cells of *Escherichia coli* strain JM109. Sequencing of the generated clone, pGEMAZI, was performed using Thermo Sequenase cycling sequencing kit (Amersham Pharmacia Biotech) with ^{33}P-labelled terminator nucleotides. The sequencing reactions were loaded on a 6% urea/polyacrylamide gel cast in a Gibco-BRL sequencing system model S2 (Life Technologies). All sequencing data were analysed using GeneWorks 2.5.1 computer software (IntelliGenetics).

Design and synthesis of plasmid constructs

The mouse antizyme cDNA [cloned into pT7Blue (Novagen)] [17], was excised with *Hin*dIII and *Eco*RI and subcloned into pALTER (Promega) that had been cut with the same enzymes. To generate the antizyme protein without ribosomal frameshifting, we deleted nt 213 (the third base in the stop codon of ORF1) by site-directed mutagenesis *in vitro*. The deletion was performed using the Altered Site kit (Promega) and a mutagenizing oligonucleotide 5'-GGTGCTCCTGTGTCCCTCAC-3'. The resulting plasmid pALTERAZ(Δ213) was used to transform competent *E. coli* strain JM109.

For expression of antizyme and AZI protein in bacteria, two plasmids (pETAZ and pETAZI) were generated by amplifying the antizyme and AZI coding regions with PCR. In this reaction gene-specific sense and antisense oligonucleotides containing *Nde*I restriction sites were used. The PCR products were subcloned into pET19b (via amplification in pGEM-T) by digestion with *Nde*I. The purified constructs were used for transformation of an *E. coli* strain (BL21DE3) that allows expression of plasmids having T7 phage promoter sequence.

For expression of the proteins in EcR-3T3 cells, four plasmid constructs were made. The coding sequences of mouse ODC, antizyme (with or without frameshift codon) and AZI were amplified by PCR. The templates were pGEMODC (a kind gift from Dr C. Kahana, The Weizmann Institute of Science, Rehovot, Israel), pALTERAZ, pALTERAZ(Δ213) and pGEMAZI. The sense primers contained *Kpn*I sites and the antisense primers *Xho*I sites. Each PCR product was digested with *Kpn*I and *Xho*I and ligated into the expression vector pIND(SP1)/V5-His A (Invitrogen), which had been digested previously with the same restriction enzymes. The coding sequences were in-frame with the C-terminal V5-detection tag, which encodes a 14-amino acid epitope derived from paramyxovirus proteins. The plasmid constructs were designated pINDODC, pINDAZ, pINDAZΔ and pINDAZI.

Purification of recombinant ODC, antizyme and AZI

Colonies of the *E. coli* strain BL21DE3, carrying pETODC, pETAZ or pETAZI (which all contained six-histidine-tag coding sequences), were inoculated into Luria-Bertani medium and grown to a D_{600} of 0.6. Isopropyl β-D-thiogalactoside was added to a final concentration of 1 mM, and the cultures were grown for 2–3 h at 30 °C. Bacteria were pelleted by centrifugation for 15 min at 20000 *g*, and resuspended in IMAC-5 buffer (20 mM Tris/HCl, 0.5 M NaCl, 10% glycerol, 5 mM imidazole and 1 mM PMSF, pH 7.9). The bacteria were then sonicated and the debris was pelleted by centrifugation. The lysate was loaded on to Ni^{2+}-charged His-Bind resin (Novagen) and flow-through was discarded. The column was washed once with IMAC-5 buffer and the His-tagged protein was eluted by the addition of IMAC-200 buffer (20 mM Tris/HCl, 0.5 M NaCl, 10% glycerol, 200 mM imidazole and 1 mM PMSF, pH 7.9). The eluate was passed through a flow cell of an Econo system controller (Bio-Rad) equipped with a recording spectrophotometer. Fractions corresponding to absorbancy peaks were pooled and concentrated using a Centricon-10 (Amicon). The ODC activity of protein mixtures containing ODC alone or ODC plus antizyme (in the presence or absence of AZI) was measured as described previously [29].

Northern-blot analysis

Total RNA was extracted from cells using the RNeasy mini kit (Qiagen). RNA was fractionated in a formaldehyde-containing

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ODC     MSSFTKDE-FDCHILDEGFTAKDILDQKINEVSSSDDKDAFYVADLGDILKKHLRWLKAL 59
AZI     MKGFIDDANYSVGLLDEGTNLGNVIDNYVYEHTLTG-KNAFFVGDLGKIVKKHSQWQTVV 59
        *  *  *  :   :****     :::*: : * : :  *:**:* *** *:*** :*   :

ODC     PRVTPFYAVKCNDSRAIVSTLAAIGTGFDCASKTEIQLVQGLGVPAERVIYANPCKQVSQ 119
AZI     AQIKPFYTVKCNSTPAVLEILAALGTGFACSSKNEMALVQELGVSPENIIFTSPCKQVSQ 119
         :: ***:**** : *::  ***:**** *:** *: *** ***  * :*:: *******

ODC     IKYAASNGVQMMTFDSEIELMKVARAHPKAKLVLRIATDDSKAVCRLSVKFGATLKTSRL 179
AZI     IKYAAKVGVNIMTCDNEIELKKIARNHPNAKVLLHIATEDNIGGEDGNMKFGTTLKNCRH 179
        *****  **:** * **** *:** **:**::*:***:*        :***:***  *

ODC     LLERAKELNIDVIGVSFHVGSGCTDPDTFVQAVSDARCVFDMATEVGFSMHLLDIGGGFP 239
AZI     LLECAKELDVQIIGVKFHVSSACKEYQVYVHALSDARCVFDMAGEFGFTMNMLDIGGGFT 239
        *** ****:::***  *** * * : : :*:*:********** * **:*::******

ODC     GSEDTKLKFEEITSVINPALDKYFPSDSGVRIIAEPGRYYVASAFTLAVNIIAKKTVWKE 299
AZI     G---TEIQLEEVNHVISPLLDIYFPEGSGIQIISEPGSYYVSSAFTLAVNIIAKKVVEND 296
        *    *:::::**:  ** * ** ***  **::**:*** ***:************** * ::

ODC     QPGSDDEDE-SNEQTFMYYVNDGVYGSFNCILYDHAHVKALLQKRPKPDEKYYSSSIWGP 358
AZI     KFSSGVEKNGSDEPAFVYYMNDGVYGSFASKLSEDLNTIPEVHKKYKEDEPLFTSSLWGP 356
        :  *  * : *:* :*:**:********    * :  :    ::*: * **  ::**:***

ODC     TCDGLDRIVERCNLPEMHVGDWMLFENMGAYTVAAASTFNGFQRPNIYYVMSRPMWQLMK 418
AZI     SCDELDQIVESCLLPELNVGDWLIFDNMGADSFHEPSAFNDFQRPAIYFMMSFSDWYEM- 415
        :** **:*** * ***::****::*:**** :    *:** **** **::**   *  *

ODC     QIQSHGFPPEVEEQDDGTLPMSCAQESGMDRHPAACASARINV 461
AZI     --QDAGITSDAMMKNFFFAP-SCIQLSQED-SFSTEA------ 448
          *  *:  :   ::    * ** * *  *   :: *
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Figure 1 Amino acid sequence alignment of AZI and ODC

Similarity was determined by comparing the side chains of the amino acid residues, using the Clustal W 1.74 sequence-alignment program. Identical amino acids are marked with an asterisk (*) and similar amino acids with a colon (:).

1% agarose gel, transferred to a Hybond-N membrane (Amersham), and hybridized to [^{32}P]dCTP random-primer-labelled cDNAs encoding mouse ODC, antizyme and AZI.

Transient transfections

Aliquots of 2×10^5 EcR-3T3 cells were seeded into 35-mm dishes and grown for 18–24 h prior to transfection. Transfections were performed using the Lipofectamin protocol (Gibco-BRL). A 1.5-μg aliquot of the construct (pINDODC, pINDAZ, pINDAZΔ or pINDAZI) was used for each transfection. After growing the transfected cells overnight, the cells were supplemented with fresh Dulbecco's minimal essential medium containing 10% FCS. They were grown for a 24-h period with 3 μM GS-E present during the last 12 or 24 h. The cells were then washed with PBS and lysed by the addition of ODC buffer (0.1 M Tris/HCl, 0.1 mM EDTA and 12.5 mM dithiothreitol, pH 7.5), containing 1% Nonidet P40. The lysates were centrifuged briefly at 13000 ***g***, and the supernatants were assayed for ODC activity as described previously [29] and subjected to Western-blot analysis (see below). The protein content was determined according to Bradford [30].

Western blot analysis

Lysates of transfected cells were loaded on a 12% polyacrylamide Readygel (Bio-Rad). Following electrophoretic separation, the proteins were transferred to a PVDF membrane (Amersham Pharmacia Biotech). All subsequent incubations and washes were made at room temperature. First the membrane was blocked in TBS-T (20 mM Tris/HCl, 0.14 M NaCl and 0.4% Tween-20) containing 1% skimmed dried milk for 30 min, which was followed by incubation for 90 min with 0.2 μg/ml anti-V5 antibody (Invitrogen) in TBS-T. The membrane was washed for 3×15 min in TBS-T and then incubated with a horseradish peroxidase-conjugated anti-mouse antibody (Amersham Pharmacia Biotech) in TBS-T containing 1% dried skimmed milk for 60 min. The membrane was washed for 3×15 min and the proteins were visualized using ECL-Plus enhanced chemiluminescence (Amersham Pharmacia Biotech).

RESULTS

We have shown previously that polyamines can down-regulate ODC activity by inducing transcription and translation of the ODC-inhibitory protein antizyme [17]. To further investigate antizyme-mediated ODC regulation we cloned and sequenced a cDNA encoding mouse AZI. The deduced amino acid sequences of mouse ODC and AZI proved to be 49% identical and 77% similar (Figure 1). The mouse AZI cDNA was amplified by PCR on the basis of sequence information derived from rat AZI cDNA [26] (sense primer) and mouse EST clones (antisense primer). The resulting PCR product contained 16 nucleotides of the 5′-untranslated region (UTR), the entire coding region and 237 nucleotides of the 3′-UTR. The coding region of mouse AZI cDNA was very similar to that of the human (93% identical) [27] and rat (96% identical) [26] AZI cDNAs (results not shown).

To determine if the cloned AZI cDNA encoded a functional protein we expressed the coding region in *E. coli*. We also expressed the coding regions of ODC and antizyme (mutated to delete the frameshift) in bacteria. After purification, the identities of all three proteins were established by SDS/PAGE (Figure 2,

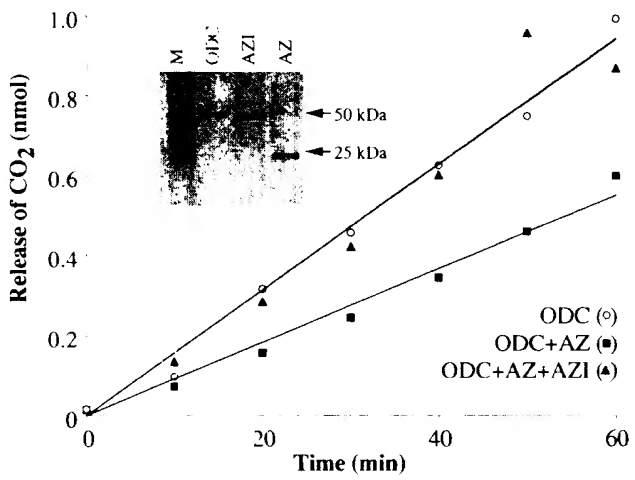

Figure 2 ODC activity in mixtures of purified recombinant proteins (ODC, antizyme and AZI)

ODC (10 ng) was incubated alone, in the presence of 50 ng of antizyme (AZ), or in the presence of antizyme and 20 ng of AZI at 37 °C for the time periods indicated. ODC activity was determined as described previously [29] (inset) The purified proteins ODC, antizyme and AZI were separated by SDS-PAGE and the proteins were visualized by staining with Coomassie Brilliant Blue G-250. The gel was dried and scanned using an Agfa Snapscan and the picture was trimmed, balanced and intensified using Adobe Photoshop 5.0 for Macintosh.

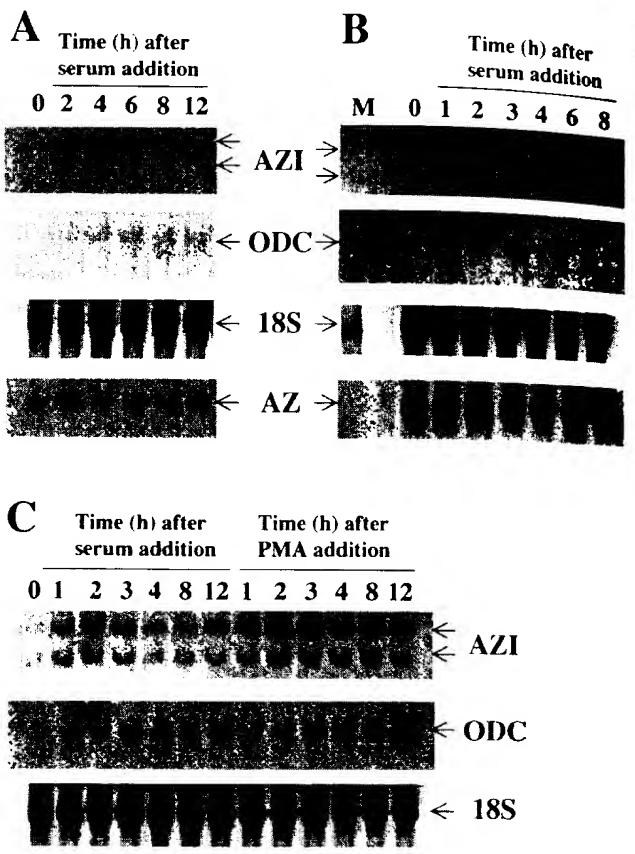

Figure 3 Effects of growth stimulation on the ODC, antizyme and AZI mRNA levels in mouse fibroblasts

EcR-3T3 (**A**), Swiss 3T3 (**B**) and Balb/c 3T3 (**C**) cells were serum-starved as described in the Materials and methods section. Serum or PMA was added, and cells were collected after the times indicated. RNA from the cells was transferred to a Nylon membrane and probed using labelled ODC, antizyme (AZ) or AZI. Equal loading of RNA was verified by ethidium bromide staining. Only the 18 S rRNA band is shown. Radioactivity was detected with a PhosphorImager (**A**) or by autoradiography (**B** and **C**).

inset). The ODC and antizyme proteins were combined and the mixture assayed for ODC activity (Figure 2). In order to obtain sufficient inhibition of ODC by antizyme we added a 5-fold excess of antizyme to the reaction mixture. This resulted in approx. 50 % reduction of the ODC activity. When the mixture also contained a 2-fold excess of AZI, the inhibition exerted by antizyme was completely counteracted (Figure 2). Neither antizyme nor AZI exhibited any ODC activity (results not shown).

It has been shown previously that the increase in cellular ODC mRNA content that follows growth stimulation of quiescent fibroblasts is due to increased transcription of the gene [31]. To determine if the cellular contents of antizyme and AZI mRNAs exhibit similar patterns of induction to ODC mRNA, we performed a Northern-blot analysis of total RNA extracted from serum-stimulated EcR-3T3 cells and Swiss 3T3 cells. Our results show that the antizyme mRNA level was high at the time of serum stimulation, and did not change significantly during growth (Figures 3A and 3B). The AZI mRNA level, on the other hand, increased rapidly with a peak within 1–2 h of the addition of serum. We also stimulated quiescent Balb/c 3T3 cells with serum or phorbol ester (PMA) and observed an increase in the AZI and ODC mRNA levels (Figure 3C). The level of expression of AZI mRNA was low, making it time-consuming to detect the mRNA with standard Northern blotting. We therefore used both autoradiography and PhosphorImager analysis in our attempts to detect the expression of AZI mRNA. We had to expose the autoradiographs for 3 weeks, or for 5 days on a PhosphorImager screen, before a signal could be seen. Unfortunately, when using the latter method, reprobing was difficult because the membranes had dried out.

As shown in Figure 2, AZI can release enzymically active ODC from a complex with antizyme *in vitro*. To test the effects of overexpression of AZI in cell culture, EcR-3T3 cells were transiently transfected with a construct containing the AZI cDNA. EcR-3T3 cells have been generated from NIH 3T3 fibroblasts by stable transfection with a plasmid expressing an ecdysone receptor. These cells permit the expression of cDNAs driven by promoters containing ecdysone-response elements [32]. Using this system, we expressed cDNAs encoding ODC, antizyme (with or without the last nucleotide in the stop codon of ORF1) and AZI. The most dramatic effect was seen when transfecting the cells with the ODC-overexpressing construct, resulting in a 7–10-fold increase in ODC activity (Figure 4, top panel). A marked decrease in endogenous ODC activity was seen upon overexpression of the mutated antizyme construct (pINDAZΔ). As expected, transfection with the antizyme construct containing the stop codon in ORF1 (pINDAZ) failed to produce a similar decrease in ODC activity. Interestingly, when the cells were transfected with the AZI-containing construct, a slight increase in ODC activity was observed, indicating reactivation of ODC from its complex with antizyme.

To verify that each construct was indeed expressed in the cells, a Western-blot analysis was performed (Figure 4, bottom panel). We used an antibody directed against the fusion tag (the V5 epitope) present in the pIND(SP1)/V5-His A vector, and thus in all transfected constructs. Figure 4 shows that ODC, antizyme and AZI were all present in the transfected cells. Because of the strong overexpression of the 46-kDa ODC protein, additional

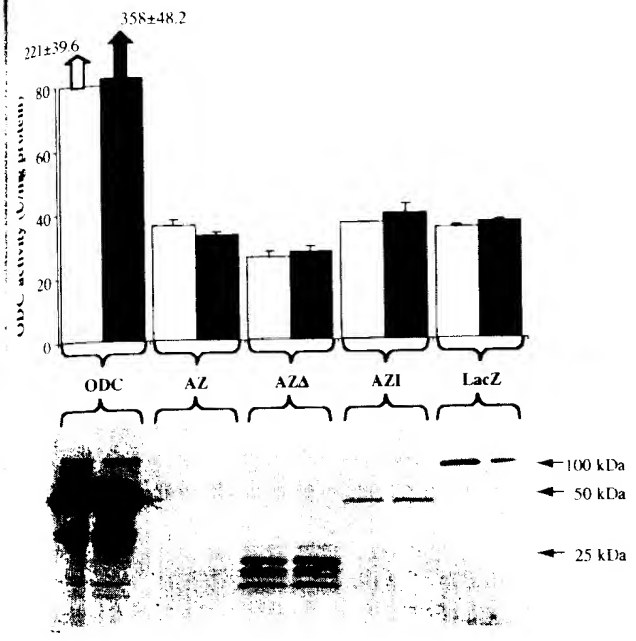

Figure 4 ODC activity in EcR-3T3 cells transiently transfected with constructs expressing ODC, antizyme or AZI, and identification of the V5-epitope-tagged proteins

EcR-3T3 cells were transfected with constructs overexpressing ODC, antizyme with frameshift codon (AZ), antizyme without frameshift codon (AZΔ) and AZI. These genes were driven by an ecdysone-inducible promoter. Transfection with pIND(SP1)-V5-His-LacZ (Invitrogen) served as a control (LacZ). The cells were induced with the ecdysone agonist GS-E for 12 (white bars) or 24 (black bars) h and lysates were assayed for ODC activity (top panel) and for the presence of V5-tagged protein by Western-blot analysis (bottom panel). Aliquots from the same lysates were used for assaying ODC activity and for Western-blot analysis. In the assay, 1 unit (U) of ODC activity was defined as the amount of enzyme catalysing the release of 1 nmol of CO_2/h. Means ± S.D. are given ($n = 3$).

bands appeared on the gel. These may represent polymerization and degradation products. As predicted, there was no detectable signal in cells transfected with a construct containing antizyme cDNA with an intact stop codon. However, in cells transfected with a construct expressing full-length antizyme protein, several additional bands were seen on the gel. These probably arise because of an additional ATG, contained in the antizyme message [17], and post-translational modifications. In cells transfected with AZI cDNA, only the predicted 45-kDa band was seen. Transfection efficiency was monitored with a construct expressing the *lacZ* gene, encoding a 105-kDa protein.

DISCUSSION

The objective of the present study was to determine whether AZI plays an active role in the growth-rate-dependent regulation of ODC [1] by interfering with antizyme. To obtain the necessary tools, we cloned and sequenced a mouse AZI cDNA containing the entire protein-coding region. The deduced amino acid sequence of the mouse AZI protein was 96% identical to that of rat AZI [26], and exhibited 49% identity to mouse ODC [33]. By overexpressing the AZI cDNA coding sequence, fused to a His-tag coding sequence, we were able to isolate a highly purified AZI that proved to be functional, i.e. it counteracted antizyme-mediated inhibition of ODC activity *in vitro*.

The expression pattern of the AZI mRNA was analysed following serum addition in order to determine if AZI is induced by growth stimulation, as is ODC. Our data show clearly that this is the case. In fact, the level of AZI mRNA increased rapidly and reached its peak level within 1–2 h of serum stimulation, i.e. at least 1 h before the peak in ODC mRNA content. The antizyme mRNA level, on the other hand, was high at the time of serum stimulation, and did not change significantly during growth. This finding suggests that antizyme protein expression is regulated mainly at the level of translation.

We have shown previously that the amount of antizyme mRNA decreases when various mouse cell lines are inhibited in their growth by polyamine depletion [17]. Nevertheless, we find that the polyamine levels in serum-starved mouse fibroblasts are sufficient to drive transcription of the antizyme gene at a rate that maintains the steady-state level of the message. Despite serum addition, the antizyme mRNA levels remained unchanged in both EcR-3T3 and Swiss 3T3 cells, at least during the period preceding DNA synthesis. At variance with the present study, Bettuzzi et al. [34] reported recently that the antizyme mRNA content of serum-starved human dermal fibroblasts changes following addition of serum. They observed a drop at 12 h and a peak at 24 h after growth stimulation. The peak coincided with a peak in the frequency of S-phase cells. These differences suggest that there are cell-specific factors that govern the transcription of the antizyme gene.

AZI mRNA reached its maximum level within 1–2 h of the addition of serum, thus preceding the peak in ODC mRNA by several hours. When Balb/c 3T3 cells were treated with the phorbol ester PMA, AZI mRNA expression was found to be stronger than when the cells were stimulated with serum. As in serum-stimulated cells, the accumulation of AZI transcripts occurred before that of ODC transcripts. However, it should be emphasized that the expression level of AZI mRNA was always much lower than that of ODC mRNA. Obviously, the regulation of the ODC and AZI genes differ significantly, despite the similarity of their amino acid coding sequences. Although it seems likely that the AZI gene is the result of an ODC gene duplication, the promoter regions of the two genes must have deviated markedly to explain their different patterns of expression. This hypothesis has not yet been corroborated by analyses of promoter sequence and efficiency.

Using Northern-blot hybridization we show that mice have two isoforms of AZI mRNA. In the rat, cDNAs encoding two isoforms have been cloned (accession numbers D50734 and D89983), but an additional AZI mRNA has been observed in Northern blots [26]. Despite their major differences in size, the two rat AZI cDNAs have identical amino acid coding regions. The size difference can be attributed to the 5′- and 3′-UTRs, both of which are longer in one of the AZI mRNAs, and may result from alternative splicing of the primary transcript and/or utilization of alternative polyadenylation signals. These possibilities remain to be analysed. It is interesting to note that the mutual relationship between the steady-state levels of the longer (4.5 kb) and the shorter (2.8 kb) AZI transcripts vary among the cell lines analysed (Figure 3). Nevertheless, both AZI mRNAs exhibit similar patterns of expression after growth stimulation, suggesting that they are transcribed from one gene, rather than two.

When comparing the sequence of the mouse AZI clone with sequences from a number of EST clones (accession numbers AA553283, AA415846, AA288344 and AA244942), we found only a few occasional mismatches. However, when comparing it with the EST clone (AA162795), from which the antisense primer was derived, an interesting feature was seen. Part of the coding sequence of this clone was missing, and upon deduction of the amino acid sequence it was seen that the coding sequence would generate a peptide that is 75 amino acids shorter than the normal

AZI protein. Interestingly, the region necessary for binding to antizyme [26] was unaffected, and the resulting protein may therefore be functional. It should be noted, however, that the coding sequence of EST clone AA162795 has not been analysed in its entire length. Therefore, in order to make the alignments we had to add N-terminal sequence derived from the mouse AZI clone. Future studies, using antibodies, should reveal whether the AZI gene gives rise to more than one isoform of the protein.

We have shown previously that rapidly proliferating cells contain only a small amount of antizyme protein [17]. To determine whether it was possible to titrate out this antizyme we overexpressed AZI and analysed whether the consequence was a release of active ODC from antizyme suppression. Indeed, when transfecting cells with a construct encoding AZI, a small increase in ODC activity was observed. This observation has recently been verified by transfection of cells with a construct containing a stronger promoter (cytomegalovirus) in front of the AZI coding sequence (results not shown). It is conceivable that the small increase in ODC activity is due to inefficient translation of the overexpressed AZI mRNA, which lacks the long UTRs that characterize the endogenous transcripts. This explanation is consistent with our Western-blot analysis, which demonstrates that although the same vector was used to drive the expression of the four different cDNAs, AZI was expressed to a much lower extent than were ODC, antizyme and LacZ. Another explanation for the small increase in ODC activity may be that AZI plays no major role in cycling cells, since the level of antizyme is already small, but is important when cells have been stimulated to enter the cell cycle from a quiescent state.

It is tempting to speculate that the AZI gene arose as a duplication of the ODC gene, and that some sequences have remained relatively unchanged during evolution because of an inherent capacity to protect ODC from antizyme-mediated degradation, especially when ODC is needed for cell growth and division. At present, no AZI gene sequences have been released in the EMBL/GenBank databases. Hence, it is not possible to compare the promoter sequences of the two genes, and the transcription factors that are involved in the regulation of AZI remain unknown. The present study provides a basis for further analyses of the intricate mechanisms of the regulation of ODC expression by antizyme and AZI. The recent discovery of a new antizyme gene [21] demonstrates additional complexity in the regulation of polyamine homoeostasis.

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